ELSEVIER

Biochimica et Biophysica Acta 1316 (1996) 149–152

BBA Biochimica et Biophysica Acta

Molecular characterization of a novel defect occurring de novo associated with erythropoietic protoporphyria

Xiuhua Wang *

Division of Pediatric Hematology, Department of Pediatrics, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, NY 10032, USA

Received 28 November 1995; accepted 14 December 1995

Abstract

A ferrochelatase (FC) mRNA lacking exon 4 was detected in a patient with erythropoietic protoporphyria (EPP). The mutation responsible for the exon skipping was a novel one: a G → C transition at the −1 position of the exon 4 donor site (nucleotide 463). The efficiency of missplicing was not 100%. The same mutation could alternatively result in exon 4 skipping or act as a missense mutation (G463 → C, predicting an Ala155 → Pro substitution), that inactivates the FC activity almost completely. Both parents were negative for the mutation and DNA fingerprinting indicated that both of them are the biological parents with 99.58% certainty. This is the first report of a de novo mutation in EPP.

Keywords: Erythropoietic protoporphyria; Ferrochelatase; Splicing mutation; Missense mutation

1. Introduction

Ferrochelatase, the last enzyme of heme biosynthesis, catalyzes the insertion of ferrous ions into protoporphyrin IX. Its deficiency is associated with EPP, a condition characterized by accumulation of excess protoporphyrin, cutaneous photosensitivity and occasional liver failure.

The molecular defects underlying EPP have been found to be very heterogenous. We have systematically screened 19 EPP families for FC mRNA with exon skipping and found 7 of them to be positive. In 6 families the responsible mutations have been previously identified [1]. The mutation in the 7th family is described in this paper. The mutation, which is associated with exon 4 skipping, was found to occur de novo and to have dual effects: it can either cause exon 4 skipping or act as a missense mutation resulting in profoundly decreased FC activity.

2. Methods

Amplification of exon 4 plus its flanking region, and sequencing of the amplified fragments were carried out as previously described [1].

Evaluation of the mutant FC was accomplished using an established expression system [2]. pHDTF20, designed for producing high levels of mature length human FC (a generous gift of Dr. H.A. Dailey), was used as a positive control for all our expression experiments. Construction of the mutant-containing expression vector pHDG463 → C is shown in Fig. 1. The FC cDNA clone bearing the G463 → C transversion was isolated and sequenced to assure that no additional mutations were present. The plasmid was then subjected to *Bam*HI/*Pst*I digestion to release a FC cDNA fragment of 849 bp encompassing the G463 → C transversion. This 849 bp fragment then replaced its counterpart in pHDTF20. A negative control vector (pHDnoE7) was similarly constructed using a FC cDNA clone with exon 7 skipping. Exon 7 is 99 bp long and therefore the *Bam*HI/*Pst*I digestion released a 750 bp fragment instead of an 849 bp fragment. An additional control vector (pHDG913 → T) containing a G913 → T transversion (predicting a Val305 → Phe substitution) [1] was also constructed and included in the expression experiments.

These FC expression vectors (pHDTF20, pHDG463 → C, pHDnoE7 and pHDG913 → T) were expressed in *E. coli* JM109 cells. Recombinant mature length FC was purified according to the published procedure [2]. Briefly, an overnight bacterial culture was collected by centrifugation and cells were disrupted by homogenization. The membrane fraction was isolated by ultracentrifugation and

* Corresponding author. Fax: +1 (212) 3055848.

PII S0925-4439(96)00003-8

solubilized by sonication. The solubilized FC was further purified by ammonium sulfate precipitation. The resuspended pellet was thus quite enriched in FC, as shown by its characteristic amber-color. The final step of the purification was protein chromatography using a Pharmacia Hi Trap Blue column. The FC was then eluted and examined on the SDS PAGE. The FC activity was determined by measuring zinc-chelating activity [3].

Blood samples were obtained with informed consent according to the ethical standard of the 1964 Declaration of Helsinki and with the approval of the Institutional Review Boards of both New York Medical College and Columbia University.

3. Results

The patient is a 10-yr-old Caucasian female with moderately severe cutaneous photosensitivity, but no clinical or laboratory evidence of abnormal liver function. Her mean erythrocyte protoporphyrin levels over the last 3 yr (6 measurements in Dr. M. Poh-Fitzpatrick's laboratory) have been 1692 ± 408 μg/dl (normal 42.9 ± 15.4). Her ferrochelatase activity determined in leukocytes was 32% of normal. There is no family history of photosensitivity. Her mother's erythrocyte protoporphyrin level was 40 μg/dl; her ferrochelatase activity was in the normal range. Her father's erythrocyte protoporphyrin level was 60 μg/dl; his ferrochelatase level was also in the normal range.

Exon 4 skipping was first indicated by hybridization of the amplified FC cDNA in the patient [1] with a probe specific for exon 4 skipping (ATT CAG AAC CCC TCA CA, the join sequence of exons 3 and 5). When the entire cDNA coding region was amplified, only one cDNA band of normal size was observed on the gel; the shorter band with exon 4 skipping could not be detected.

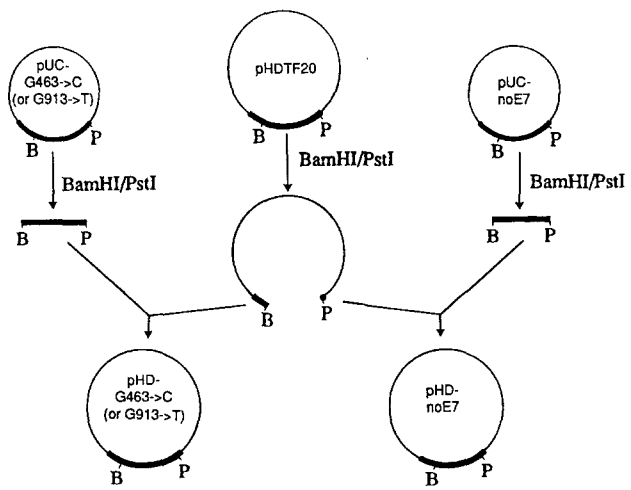

Fig. 1. Construction of FC expression vectors. pHDTF20 = expression vector designed by H. Dailey to produce mature length human FC. pUCG463 → C (or pUCG913 → T) = pUC clone containing FC cDNA with G463 → C (or G913 → T) substitution; pUCnoE7 = pUC clone containing FC cDNA with exon 7 skipping. From these pUC plasmids, an FC cDNA fragment bearing G463 → C (or G913 → T) substitution and an FC cDNA fragment with exon 7 skipping were respectively released by *Bam*HI/*Pst*I digestions. These were next ligated to pHDTF20 linearized by the same two restriction enzymes. The symbols used in the figure represent: thick line, FC cDNA; B, the *Bam*HI site; and P, the *Pst*I site.

Exon 4 skipping distorts the FC reading frame: a premature stop codon renders the aberrant mRNA lacking exon 4 very unstable. To confirm the skipping, primers were designed according to the exon 3 and 5 sequences to capture more aberrant mRNA for amplification. As expected, an extremely faint shorter band, presumably lacking exon 4, was seen on the gel along with the normal size cDNA. The shorter band was then re-amplified and directly sequenced. The exon 3 sequence was found to directly join the exon 5 sequence without any insertion or addition, thus confirming the absence of exon 4. All other sequences in this aberrant transcript appeared normal. The mutation responsible was identified as G463 → C, the −1 position of the exon 4 donor site. This is a hot spot (the −1 position of an exon donor site) for splicing mutations, as many similar mutations have been reported in other genes [4–6]. The patient is heterozygous for the mutation.

Skipping of exon 4 does not occur with 100% efficiency, as the mRNA bearing the G463 → C transversion (predicting an Ala155 → Pro substitution) is present at low levels. The FC cDNA bearing the G463 → C transvertion was thus cloned and isolated (Fig. 1). The fragment bearing the transversion was then excised and used to replace its normal counterpart in the FC expression vector pHDTF20. For a negative control, the FC cDNA lacking exon 7 was similarly cloned into pHDTF20 (Fig. 1, pHDnoE7). Another FC missense mutation (G913 → T, predicting a Val305 → Phe substitution), characterized by us previously [1], was also cloned into pHDTF20 as an additional control (pHDG913 → T). After transformation, the recombinant FC was purified according to the published procedure and examined by SDS-gel electrophoresis [2]. A single band of the recombinant FC (about 40 kDa) was observed for the control vector pHDTF20, in perfect agreement with the published result [2]. An identical band was observed for the mutation-bearing vectors pHDG463 → C, pHDG913 → T and pHDnoE7, but not for JM109 host cells without external transformant (Table 1). These data, therefore, suggested that the mutated FC was synthesized from pHDG463 → C in the expression experiments. The enzyme activity (U/ng protein) was measured on cell homogenates and the purified recombinant FC. Compared to the control pHDTF20, the mutation-bearing vector pHDG463 → C showed < 3% FC activity, a level comparable to that of both the negative control pHDnoE7 and of JM109 host cells without external transformant (Table 1). Repeated expression experiments gave consistent results. pHDG463 → C was also sequenced to confirm that no additional mutations were introduced during vector construction. These data, therefore, suggest that the G463 → C

Table 1
Relative FC activity of JM109 cells transformed with various vectors

Cells transformed with vector	Mutation	40 kDa protein band	Activity (%) [a]
pHDTF20	–	+	100
pHDG463 → C	G463 → C (Ala155 → Pro)	+	< 3
PhDG913 → T	G913 → T (Val305 → Phe)	+	120
pHDnoE7	Exon 7 skipping	+	< 3
No vector	–	–	< 3

[a] Enzyme activity of pHDTF20 transformed cells, expressed in U/ng protein, was considered as 100%

(Ala155 → Pro) transversion is associated with inactivation of the FC activity.

To study the inheritance pattern of the mutation, allele-specific oligonucleotide hybridization was performed, but it failed to detect the mutation in either parent. Exon 4 plus its flanking region was also amplified and sequenced for both parents, but only normal sequences were found. On the other hand, DNA fingerprinting indicated that both mother and father are the biological parents with 99.58% certainty [7]. These findings demonstrate that the mutation in the child occurred de novo. This is the first time that a de novo mutation has been observed in EPP.

4. Discussions

The mutation described in this patient has two distinct functional effects.

- On the one hand, it acts as a splicing mutation causing exon 4 skipping. The skipping of exon 4 distorts the reading frame, generating a premature stop codon at nucleotides 713–715. This in turn gives rise to an inactive truncated protein of 124 amino acids. Transcripts bearing premature stop codons are often very unstable: this is probably the reason for the low level of FC mRNA lacking exon 4 observed.
- On the other hand, when the mutant allele splices correctly, a mutant FC with a missense mutation (Ala155-Pro) that has very little catalytic activity is generated. The amino acid (Ala155) is conserved in mouse and yeast FC; thus, it appears likely that it plays a key role in the enzyme function.

Consensus sequences functioning in RNA splicing involve both exon and intron sequences. When mutations occur on the exon region, they could, depending on specific mutations, affect both the splicing and coding of the protein. The same phenomenon was also observed for another FC mutation and other genes [1,8]. We described previously a G913 → T transversion on the + 1 position of the exon 9 splicing acceptor site of the FC gene [1]. The transversion primarily caused exon 9 skipping, but a transcript bearing the G913 → T transversion (predicting a Val305 → Phe substitution) was also observed. Expression experiment with FC cDNA bearing the G913 → T transversion showed no decrease in FC activity (Table 1). Another such example is a G244 → T transversion (predicting a Val82 → Phe substitution) associated with congenital erythropoietic porphyria on uroporphyrinogen III synthase gene [8]. The mutation occurred at the − 1 position of the exon donor splicing site and resulted in exon skipping in about 50% of the transcripts encoded by this allele. The other half of the transcripts, bearing the missense mutation, gave rise to an enzyme with about 35% of normal activity.

The exon 4 skipping was initially detected by hybridization of amplified FC cDNA with an oligonucleotide probe specific for exon 4 skipping (the exons 3 and 5 joining sequence that results from exon 4 skipping). The use of these newly created joining sequences as markers has been shown previously by us to be a very sensitive and efficient method for detecting transcripts with exon skipping [1], especially those that distort the reading frame, yielding very unstable transcripts.

More than a dozen EPP mutations have been identified [9–22]. Only a few mutations were reported in more than one family. Todd et al. [16] found 3 of 6 EPP families in Northern Ireland sharing a single base pair deletion in exon 1 and we found 3 EPP families sharing a splicing and nonsense mutation at exon 10 [14]. The majority of the families screened by us to date remain negative for all mutations currently identified.

In the patient's family, both parents were negative for the mutation: this is the first de novo mutation described in EPP.

Molecular defects underlying EPP appear extremely heterogenous, since most reported familles have a unique mutation. This may reflect the fact that EPP is usually a mild disease and therefore very little evolutionary pressure is exerted on it.

Acknowledgements

Special thanks to Dr. S. Piomelli for his support and direction, Dr. M. Poh-Fitzpatrick for her support and allowing the use of the patient's biochemical data, Dr. H.A. Dailey for giving us the expression vector pHDTF20, T. Chen for technical assistance, and C. Seaman for help with the manuscript. This work was supported by Grant #HL48996 and #AR18549 from the National Institutes of Health, Bethesda, MD, USA.

References

[1] Wang, X., Poh-Fitzpatrick, M., Chen, T., Malavade, K., Carriero, D. and Piomelli, S. (1995) Biochim. Biophys. Acta 1271, 358–362.
[2] Dailey, H.A., Sellers, V.M. and Dailey, T.A. (1994) J. Biol. Chem. 269, 390–395.

[3] Nunn, A.V.W., Norris, P., Hawk, J.L.M. and Cox, T.M. (1988) Anal. Biochem. 174, 146–150.
[4] Akli, S., Chelly, J., Mezard, C., Gandy, S., Kahn, A. and Poenaru, L. (1990) J. Biol. Chem. 265, 7324–7330.
[5] Berg, L.-P., Grundy, C.B., Thomas, F., Millar, D.S., Green, P.J., Slomski, R., Reiss, J., Kakkar, V.V. and Cooper, D.N. (1992) Genomics 13, 1359–1361.
[6] Grandchamp, B., Picat, C., de Rooij, F., Beaumont, C., Wilson, P., Deybach, J.C. and Nordmann, Y. (1989) Nucleic Acids Res. 17(16), 6637–6649.
[7] Helminen, P., Sajantila, A., Johnson, V., Lukka, M., Ehnholm, C. and Peitonen, L., (1992) Mol. Cell. Probes 6, 21–26.
[8] Xu, W., Warner, C.A. and Desnick, R.J. (1995) J. Clin. Invest. 95, 905–912.
[9] Lamoril, J., Boulechfar, S., de Verneuil, H., Grandchamp, B., Nordmann, Y. and Deybach, J.C. (1991) Biochem. Biophys. Res. Commun. 181, 594–599.
[10] Brenner, D.A., Didier, J.M., Frasier, F., Christensen, S.R., Evans, G.A. and Dailey, H.A. (1992) Am. J. Hum. Genet. 50, 1203–1210.
[11] Nakahashi, Y., Fujita, H., Taketani, S., Ishida, N., Kappas, A. and Sassa, S. (1992) Proc. Natl. Acad. Sci. USA 89, 281–285.
[12] Nakahashi, Y., Miyazaki, H., Kadota, Y., Naitoh, Y., Inoue, K., Yamamoto, M., Hayashi, N. and Taketani, S. (1993) Hum. Mol. Genet. 2, 1069–1070.
[13] Nakahashi, Y., Miyazaki, H., Kadota, Y., Naitoh, Y., Inoue, K., Yamamoto, M., Hayashi, N. and Taketani, S. (1993) Hum. Genet. 91, 303–306.
[14] Wang, X., Poh-Fitzpatrick, M., Carriero, D., Ostasiewicz, L., Chen, T., Taketani, S. and Piomelli, S. (1993) Biochim. Biophys. Acta 1181, 198–200.
[15] Sarkany, R.P., Whitcombe, D.M. and Cox, T.M. (1994) J. Invest. Dermatol. 102, 481–484.
[16] Todd, D.J., Hughes, A.E., Ennis, K.T., Ward, A.J., Burrows, D. and Nevin, N.C. (1993) Hum. Mol. Genet. 2, 1495–1496.
[17] Wang, X., Poh-Fitzpatrick, M. and Piomelli, S. (1994) Biochim. Biophys. Acta 1227, 25–27.
[18] Schneider-Yin, X., Schafer, B.W., Mohr, P., Burg, G. and Minder, E.I. (1994) Hum. Genet. 93, 711–713.
[19] Magness, S.T., Tugores, A., Christensen, S.R., Wagner-Mcpherson, C., Evans, G.A., Naylor, E.W. and Brenner, D.A. (1994) Hum. Mol. Genet. 3, 1695–1697.
[20] Sarkany, R.P., Alexander, G.J. and Cox, T.M. (1994) Lancet 344, 958–959.
[21] Schneider-Yin, X., Schäfer, B.W., Tönz, O. and Minder, E.I. (1995) Hum. Genet. 95, 391–396.
[22] Sarkany, R.P.E., Alexander, G.J.M. and Cox, T.M. (1994) Lancet 343, 1394–1396.